Endogenous hydrogen sulphide mediates the cardioprotection induced by ischemic postconditioning

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Yong QC, Lee SW, Foo CS, Neo KL, Chen X, Bian JS. Endogenous hydrogen sulphide mediates the cardioprotection induced by ischemic postconditioning. Am J Physiol Heart Circ Physiol 295: H1330–H1340, 2008. First published July 25, 2008; doi:10.1152/ajpheart.00244.2008.—The present study aimed to investigate the role of hydrogen sulphide (H2S) in the cardioprotection induced by ischemic postconditioning and to examine the underlying mechanisms. Cardiodynamics and myocardial infarction were measured in isolated rat hearts. Postconditioning with six episodes of 10-s ischemia (IPostC) significantly improved cardiodynamic function, which was attenuated by the blockade of endogenous H2S production with d,l-propargylglycine. Moreover, IPostC significantly stimulated H2S synthesis enzyme activity during the early period of reperfusion. However, d-l-propargylglycine only attenuated the IPostC-induced activation of PKC-ε and PKC-α but not that of PKC-β, Akt, and endothelial nitric oxide synthase (eNOS). These data suggest that endogenous H2S contributes partially to the cardioprotection of IPostC via stimulating PKC-α and PKC-ε. Postconditioning with six episodes of a 10-s infusion of NaHS (SPostC) or 2 min continuous NaHS infusion (SPostC2) stimulated activities of Akt and PKC, improved the cardiodynamic performances, and reduced myocardial infarct size. The blockade of Akt with LY-294002 (15 μM) or PKC with chelerythrine (10 μM) abolished the cardioprotection induced by H2S postconditioning. SPostC2, but not SPostC, also additionally stimulated eNOS. We conclude that endogenous H2S contributes to IPostC-induced cardioprotection. H2S postconditioning confers the protective effects against ischemia-reperfusion injury through the activation of Akt, PKC, and eNOS pathways.

Akt; protein kinase C; hydrogen sulphide; endothelial nitric oxide synthase

EVER SINCE ITS DISCOVERY in 1986 by Murry et al. (27), ischemic preconditioning (IPC) has been positioned as one of the most potent cardioprotective mechanisms against ischemia-induced injuries. Despite the promising effect of IPC, it has a major limitation in that brief ischemia maneuver or its mimetic, which can trigger pharmacological preconditioning, has to be applied before the index ischemia insult. This has lead to the introduction of the concept ischemic postconditioning (IPostC) by Na et al. (29) and Zhao et al. (59). IPostC is defined as the phenomenon where rapid intermittent interruptions of blood flow in the early phase of reperfusion resulted in a reduced myocardial injury (60). This maneuver has produced promising protection against ischemia-reperfusion injury in mice (21), rats (22), rabbits (55), dogs (59), and in human patients (38).

The main purpose of introducing shunting during the initial reperfusion phase after ischemia is to disrupt a process known as lethal reperfusion injury. Lethal reperfusion injury occurs as a result of a sudden reflow of blood into the heart, which leads to an abrupt change in the vascular environment. This results in detrimental events, which subsequently lead to endothelial and vascular dysfunction, metabolic dysfunction, contractile dysfunction, dysrhythmias, and eventually myocytes necrosis and apoptosis (60). The mechanisms giving rise to the protective effect of IPostC constitute two arms: passive and active (45). The passive arm of IPostC refers to the mechanical events and cellular events directly resulted from the IPostC maneuver (46). The mechanical events interrupt the sudden onset of the full flow reperfusion responsible for lethal reperfusion injury such as the negative modulation of coronary perfusion pressure, whereas the cellular events involve the increased nitric oxide (NO) release due to better endothelial cell survival and the preservation of their functions (46). In addition to the passive arm, IPostC treatment activates several prosurvival kinases such as Akt (44, 62), protein kinase C (PKC) (39, 57), extracellular signal-regulated kinase 1/2 (ERK1/2) (6), and inhibits c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase (39). These molecular events constitute the active arm of IPostC.

Hydrogen sulphide (H2S) has been positioned as the third gasotransmitter followed after NO and carbon monoxide (48). In the cardiovascular system, H2S was generated predominantly by cystathionine-γ-lyase (CSE) (4, 41, 48). CSE activity has been detected in vascular smooth muscle (58) and the heart (13). In addition, ∼46 μM H2S was detected in rat serum (58), whereas ∼52 μM H2S was detected in human serum (18). As such, the heart was constantly bathed in a considerable amount of H2S generated by the cardiac myocytes locally and from the blood. Interestingly, several studies have suggested a correlation between CSE activity, H2S concentration, and myocardial injury. In human patients, it was reported that plasma H2S concentration was significantly lower in patients with coronary heart disease (18). Besides, the concentration of endogenous H2S in plasma and myocardial tissue was significantly decreased in isoproterenol-induced myocardial injury (12, 56). In addition, the concentration of H2S detected in the medium of cardiac myocytes was decreased upon ischemia treatment (2). As such, the level of endogenous H2S might be tightly regulated in the heart, and ischemia insult could induce a significant change in the production of endogenous H2S by CSE.

H2S has been shown to protect the heart from myocardial ischemia-reperfusion in various studies (10, 19, 36, 63). Recent work from our laboratory has shown that H2S preconditioning produced cardiac protective effects similar to that induced by IPC (2, 30). In addition, exogenous H2S was shown to activate several prosurvival kinases such as ERK1/2 (51, 52, 61), Akt

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Although the complete signaling mechanisms of H$_2$S remain to be clarified, the general understanding on the cardioprotective effect of H$_2$S to date has led us to hypothesize that endogenous H$_2$S may contribute to the protective effect of IPostC. As such, the aim of our study was to examine whether endogenous H$_2$S plays a role in mediating the active arm of IPostC via the activation of prosurvival kinases. In addition, the role of exogenous H$_2$S to serve as a potential candidate to trigger pharmacological postconditioning was also studied.

**MATERIALS AND METHODS**

The study protocol was approved by the Institutional Animal Care and Use Committees of National University of Singapore and conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

**Chemicals.** NaHS and d,L-propargylglycine (PAG) were purchased from Sigma Chemical. Chelerythrine (Che) chloride and LY-294002 (LY) were purchased from Calbiochem (Darmstadt, Germany). Polyclonal anti-phospho (p)-Akt rabbit IgG and polyclonal anti-total-Akt rabbit IgG were purchased from Cell Signaling Technology. Polyclonal anti-PKC-ε rabbit IgG antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Chelerythrine chloride and LY was dissolved in dimethyl sulfoxide (DMSO) and added to the Krebs bicarbonate buffer such that the final DMSO concentration was less than 0.1%. All other chemicals were dissolved in deionized water.

As used in numerous publications, NaHS, a donor of H$_2$S, was employed in these experiments since its use allows for a better determination of the concentration of H$_2$S in solution than bubbling H$_2$S gas. NaHS dissociates to Na$^+$/H$_2$S$^-$ and HS$^-$/H$_2$S in solution. Thereafter, HS$^-$/H$_2$S$^-$ associates with H$^+$ and produces H$_2$S. Approximately one-third of the H$_2$S in aqueous solution exists in the undissociated form (H$_2$S) at 20°C. At 37°C, the undissociated form of H$_2$S is around 18.5% (8), i.e., $\sim$18.5 μM when 100 μM NaHS was applied, which is well tolerated by the heart.

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![Graphs and diagrams related to the study](https://example.com/figure1.png)

**Fig. 1.** Activity of hydrogen sulphide (H$_2$S)-generating enzymes with and without postconditioning with 6 episodes of 10-s ischemia (IPostC) and the effect of d,L-propargylglycine (PAG) on cardiodynamic function. **A**: experimental protocols. White field, Krebs solution; black field, no-flow ischemia. PAG, a H$_2$S synthesis inhibitor, was given 15 min before, during, and 2 min after ischemia treatment. **B**: effect of IPostC on the activity of H$_2$S synthesis enzymes with and without IPostC. Mean data showing the H$_2$S production produced by the H$_2$S-generating enzyme in the homogenates of rat ventricular tissue in the presence of substrate and cofactors are shown. 0 time group served as a negative control, representing the start of the experiment when no H$_2$S is produced. The next 4 bars show the production of H$_2$S after 30 min. All values are means ± SE (n = 8). #P < 0.05, vehicle postconditioning group (VPostC) vs. control; **P < 0.01, VPostC vs. IPostC; †P < 0.05, IPostC vs. IPostC + PAG. **C** and **D**: effect of PAG on cardiodynamic function. C: representative tracings showing the mechanical performance of isolated heart during perfusion of 2 mM PAG for 40 min (bottom) compared with Kreb’s solution only (top). **D**: mean data of left ventricular developed pressure (LVDP) showing that 2 mM PAG alone did not alter the LVDP significantly. I/R, ischemia-reperfusion.
within the range of physiological concentrations in rat plasma (58). It is notable that 100 μM NaHS does not alter the pH of the buffers (9). For this reason, NaHS has been widely used for studies on H2S (7, 9).

Isolated rat heart perfusion model. Adult male Sprague-Dawley rats (8 wk; 200–250 g) were anesthetized by an intraperitoneal (ip) administration of pentobarbital (60 mg/kg). Heparin (1,000 international units ip) was administered to prevent coagulation during removal of the heart. A central thoractotomy was performed, and the heart was rapidly excised and placed in ice-cold buffer. The heart was mounted onto a Langendorff apparatus via aortic root and perfused with modified Krebs bicarbonate buffer containing (in mM) 118 NaCl, 5.4 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 25 NaHCO3, and 11 glucose (pH 7.4 at 37°C).

The electrocardiogram (ECG) of the isolated heart was monitored and recorded with two electrodes hooked to the apex and the aorta, respectively, as described previously (2). Isolated hearts were allowed to stabilize for 10 min. Any heart that exhibited arrhythmias during this period was discarded. Isolated rat hearts with normal ECGs were randomly assigned to the experiment groups.

Measurement of cardiodynamic functions. Cardiodynamic parameters were measured with a pressure transducer connected to a PowerLab system (ADInstruments). An incision was made in the left atrium, and a fluid-filled latex balloon connected to the pressure transducer was inserted and positioned in the left ventricular cavity for the continuous assessment of cardiodynamic function. The balloon was initially inflated to an end-diastolic pressure of 5–10 mmHg, and thereafter the balloon volume was held constant. The hearts were perfused at a constant flow rate of 12 ml/min. Cardiodynamic data were analyzed using a Data Acquisition System (PowerLab System; ADInstruments). Left ventricular end-diastolic pressure (LVeDP) was represented by the minimum pressure recorded during diastoles, left ventricular developed pressure (LVPDP) was calculated as the difference between left ventricular systolic pressure and left ventricular diastolic pressure, contractility (+dP/dt) was represented by the maximum gradient during systoles, and compliance (−dP/dt) was represented by the minimum gradient during diastoles.

Measurement of myocardial infarction size. Regional ischemia was induced by ligating the left anterior descending main coronary artery for 40 min. The ligation was released during the 2-h reperfusion. After 2 h reperfusion, the infarct-risk volume ratio was determined. As described previously (14), the heart was stained by slowly infusing 1 ml of Evan’s blue (3% wt/vol in phosphate-buffered saline) via the aorta, followed by perfusion with Krebs bicarbonate buffer to wash out unbound stains. The heart was then immediately removed from the perfusion apparatus, weighed, and stored overnight at −20°C. The frozen heart was thereafter cut into five or six transverse sections (~2 mm in thickness) across the long axis, stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4), for 20 min at 37°C, and then fixed in 10% Formalin overnight. The size of the myocardial infarction (appearing as pale color) was quantified by ImageJ (1.32j; NIH) and calculated as the percentage of area at risk, which is the area not stained by Evan’s blue.

Fig. 2. Effect of IPostC on cardiodynamics in the presence and absence of PAG, a H2S synthesis inhibitor. A: experimental protocols. White field, Krebs solution; black field, no-flow ischemia. PAG was given 15 min before, during, and 2 min after ischemia treatment. B: representative tracings showing the mechanical performance of isolated rat hearts during 40 min ischemia and subsequent 1 h reperfusion in VPostC, IPostC, and IPostC + PAG groups. C: mean data of left ventricular end-diastolic pressure (LVeDP; top left), LVPDP (top right), contractility (+dP/dt; bottom left), and compliance (−dP/dt; bottom right). All values are means ± SE (n = 6). *P < 0.05, VPostC vs. IPostC; #P < 0.05, IPostC vs. IPostC + PAG.
**Experimental protocol.** Isolated rat hearts were perfused and stabilized for at least 20 min before data recording. Global ischemia was mimicked by stopping the perfusion of Krebs bicarbonate buffer, i.e., perfusion rate = 0 ml/min. The hearts were randomly divided into nine groups according to the perfusion protocol as shown in Fig. 1A, 2A, 5A, 9A, and 10A. The vehicle postconditioning group (VPostC) and ischemic postconditioning group (IPostC) served as the negative and positive controls in this study. In VPostC and IPostC groups, hearts experienced 20 min equilibration followed by 40 min global ischemia. Upon reperfusion, IPostC hearts were treated with six cycles of 10-s reperfusion and 10-s no-flow ischemia (total intervention time of 2 min), whereas VPostC hearts did not receive any additional treatment. In the IPostC + PAG group, hearts were pre-treated with 2 mM PAG, a CSE inhibitor, 15 min before, during, and 2 min after ischemia treatment (Figs. 1A and 2A). Two protocols of H$_2$S postconditioning were adopted: SPostC group hearts were treated with six cycles of 10-s reperfusion and 10-s 100 µM NaHS, a H$_2$S donor, intermittently, whereas SPostC2 group hearts were treated with 100 µM NaHS for 2 min continuously (Fig. 5A). NaHS treatments were performed by directly injecting 100 µM NaHS into perfusing Krebs bicarbonate buffer to avoid flow interruption caused by the switching of stopcocks. In four parallel groups (SPostC-LY, SPostC2-LY, SPostC-Che, and SPostC2-Che), hearts were treated with 15 µM LY [phosphatidylinositol 3-kinase (PI3K)/Akt inhibitor] or 10 µM Che (PKC inhibitor) 10 min before, during, and 5 min after ischemia insults (Fig. 9A and 10A).

**Measurement of H$_2$S synthesis enzymes activity.** The tissue H$_2$S production rate was measured essentially as described previously (49, 56). Briefly, snap-frozen 100 mg of rat ventricular tissues from VPostC, IPostC, and IPostC + PAG groups were homogenized in 1 ml of 100 mM potassium phosphate buffer (pH 7.4). The assay mixtures (500 µl) contained 460 µl of tissue homogenate, 100 mM 1-cysteine, and 2 mM pyridoxal 5’-phosphate. Incubation was carried out in tightly sealed Eppendorf vials. After incubation (37°C, 30 min), zinc acetate (1% wt/vol, 250 µl) was added to trap the generated H$_2$S followed by trichloroacetic acid (10% wt/vol, 250 µl) to stop the reaction. N,N-Dimethyl-p-phenylenediamine sulfate (20 mM, 133 µl) and FeCl$_3$ (30 mM, 133 µl) was next added and centrifuged under 14,000 g. After 10 min, absorbance at 670 nM of 200-µl aliquots of the supernatant was determined. The H$_2$S concentration of samples was calculated using a calibration curve of NaHS (3.125–200 µM), and the results were expressed as nanomoles of H$_2$S produced per milligram of soluble protein per 30 min. Protein was determined with NanoDrop Spectrophotometer (ND-1000, NanoDrop technology).

**Western blot analysis.** After 1 h reperfusion, the ventricular tissues of the heart that had undergone global ischemia were freeze-clamped in liquid nitrogen and stored at −80°C for further analysis. The phosphorylation state of Akt and endothelial NO synthase (eNOS), and translocation of PKC-α, PKC-δ, and PKC-ε, was assessed with standard Western immunoblotting. Briefly, 25 mg of tissues were minced and homogenized in ice-cold lysis buffer containing (in mM) 25 Tris-HCl (pH 7.5), 150 NaCl, 5 EDTA, 10 NaF, 1 Na$_3$VO$_4$, 1% Nonidet P-40 and 0.4% deoxycholic acid supplemented with protease inhibitor cocktail tablet (Roche Diagnostics, Penzberg, Germany). A cell fractionation technique was adopted from the literature (50). Homogenates were centrifuged at 1,000 g for 10 min. The cytosolic fraction of the proteins was obtained by collecting the supernatant and centrifuged at 16,000 g to maximize protein extraction. The membrane fraction was obtained by treating the pellet with lysis buffer supplemented with 1% Triton X followed by centrifugation at 16,000 g. Protein concentrations were determined using the modified Lowry method. Proteins were denatured with SDS sample buffer, and epitoxes were exposed by boiling the protein samples at 100°C for 5 min. Equal amounts of proteins were loaded and separated by electrophoresis on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was first probed with the primary antibody that recognizes p-Akt (serine 473), total Akt, p-eNOS (serine 1177), total eNOS, PKC-α, PKC-δ, or PKC-ε and then with a horseradish peroxidase-conjugated goat-anti-rabbit IgG secondary antibody. Immunoreactivity was detected using the enhanced chemiluminescence method. Protein levels were normalized to the VPostC group.

**Statistical analysis.** All values were expressed as means ± SE. Cardiodynamic data were assessed with one-way ANOVA followed by Tukey’s post hoc test. Immunoreactivity of each protein of interest was normalized to VPostC and compared among the groups by ANOVA and subsequent Newman-Keuls post hoc test. Differences were considered statistically significant when P < 0.05.

**RESULTS**

**Activity of H$_2$S synthesis enzymes in ischemia-reperfusion with and without IPostC treatment.** The experimental protocols are shown in Fig. 1A. H$_2$S-generating enzyme activities in the heart tissues were measured at the end of six cycles of postconditioning treatment in the IPostC group or after 2 min of reperfusion in the VPostC group. As shown in Fig. 1B, H$_2$S synthesis enzyme activity was significantly decreased in the VPostC group, suggesting that ischemia may inhibit endogenous H$_2$S production. This is consistent with our previous findings that ischemia decreases H$_2$S production in isolated...
cardiomyocytes (2, 30). However, the decreased enzyme activity was dramatically reversed and enhanced by IPostC, suggesting that IPostC treatment can strongly stimulate the production of endogenous H$_2$S, which may serve as a mediator for the cardioprotective effects of IPostC.

It was also found that the effect of IPostC on H$_2$S-generating enzyme activity was blocked by PAG (Fig. 1B), confirming that PAG can serve as an inhibitor of CSE in our experimental model. To test whether PAG can be safely used in the functional studies, we also examined its effect on the cardiodynamic function in the isolated rat hearts. As shown in Fig. 1, C and D, the administration of PAG (2 mM) for 40 min did not impair LVDP. PAG was therefore employed in the following experiments to test the involvement of H$_2$S in the cardioprotection of IPostC.

**Role of endogenous H$_2$S in the cardioprotection induced by IPostC.** Cardiodynamic function was measured to test the involvement of endogenous H$_2$S in the cardioprotection induced by IPostC. The experimental protocols are shown in Fig. 2A and described in MATERIALS AND METHODS. As shown in Fig. 2, B and C, IPostC significantly protected the heart by resuming the functional performance after ischemia compared with that of VPostC when assessed with LVEdP, LVDP, and $+dP/dt$. The inhibition of endogenous H$_2$S synthesis with 2 mM PAG from 15 min before up to 2 min after ischemia significantly diminished the cardioprotective effect of IPostC by elevating LVEdP and reducing both LVDP and $+dP/dt$. However, no significant change in $-dP/dt$ was observed, implying a minor role played by endogenous H$_2$S in heart relaxation.

**Role of endogenous H$_2$S in the activation of PKC isoforms triggered by IPostC.** PKC is an important trigger/banker in the cardioprotection induced by IPostC (34, 57). The PKC family consists of at least 10 isoforms, among which PKC-ε, PKC-ζ, and PKC-δ are the prominent isoforms expressed in the heart (25). We therefore examined whether endogenous H$_2$S mediates the activation of these three PKC isoforms during IPostC. As shown in Fig. 3, the translocation of different PKC isoforms (α, δ, and ε) from cytosol to membrane was observed in the IPostC treatment group, indicating IPostC stimulates these PKC isoforms. The inhibition of endogenous H$_2$S synthesis with PAG significantly reversed the translocation of PKC-α and PKC-ε but not PKC-δ (Fig. 3). These data suggest that endogenous H$_2$S played a part in IPostC-triggered activation of PKC-ε and PKC-α.

**Role of endogenous H$_2$S in the activation of Akt and eNOS triggered by IPostC.** Since IPostC was shown to trigger cardioprotection partly via the activation of prosurvival kinase Akt (44, 62) and eNOS (44), we further examined whether the inhibition of endogenous H$_2$S production could reverse the phosphorylation of Akt and eNOS upon IPostC treatment. As shown in Fig. 4, IPostC significantly induced the phosphorylation of Akt (Fig. 4A) and eNOS (Fig. 4B). This is consistent with the previous findings from other groups (44, 62). However, the inhibition of endogenous H$_2$S synthesis with PAG failed to attenuate the IPostC-induced phosphorylation of Akt (Fig. 4A) and eNOS (Fig. 4B), suggesting that the activation of both enzymes by IPostC was not solely via endogenous H$_2$S.

H$_2$S postconditioning improves the cardiodynamic performance of isolated perfused rat heart after ischemia. This series of experiments was designed to determine whether postconditioning with exogenous H$_2$S was also able to produce cardioprotection. NaHS reperfusion was used to substitute the six episodes of ischemia-reperfusion in IPostC. As shown in Fig. 5A, two pharmacological postconditioning protocols that were commonly adopted by other groups (24, 43) were used in the present study. Since NaHS at 100 μM exhibited the maximal cardioprotective effect from ischemia-induced injury in both cardiac myocytes (30) and isolated rat hearts (17), 100 μM was therefore chosen to observe the cardioprotection of NaHS postconditioning in the following experiments. In SPostC group, hearts received six cycles of 10-s reperfusion and 10-s NaHS infusion after ischemia, whereas in SPostC2, NaHS (100 μM) was given for 2 min continuously after ischemia. Figure 5, B and C, showed that both SPostC and SPostC2 treatment significantly improved the cardiodynamics including LVEdP, LVDP, and $+dP/dt$ during reperfusion after ischemia. The cardioprotective effect was comparable with that of IPostC.

H$_2$S postconditioning limits myocardial infarct size of isolated perfused rat heart. To further confirm the cardioprotective effects of SPostC, infarct size was assessed in VPostC, SPostC, and SPostC2 groups. Hearts received SPostC and...
SPostC2 treatment displayed 71% and 72% reductions, respectively, in infarct size per area at risk (SPostC, 12.7 ± 5%; and SPostC2, 12.4 ± 3%) compared with that in vehicle group (44.1 ± 5%; Fig. 6). These data confirm that H2S may serve as a possible trigger for pharmacological postconditioning.

H2S postconditioning activates Akt, eNOS, and PKC. The signaling mechanisms underlying the cardioprotection of H2S postconditioning were also examined. We first determined the involvement of PKC isoforms (α, δ, and ε) in the cardioprotection triggered by H2S postconditioning. SPostC translocated all three isoforms of PKC; however, SPostC2 only induced PKC-δ activation (Fig. 7), implying that SPostC and SPostC2 may induce cardioprotection via different signaling mechanisms.

Since both Akt and eNOS mediate the cardioprotection of IPostC, the phosphorylation of Akt and eNOS was also examined. When compared with VPostC, both SPostC and SPostC2 treatment induced significant phosphorylation of Akt (Fig. 8A). Interestingly, SPostC2, but not SPostC, also induced the phosphorylation of eNOS (Fig. 8B).

Roles of Akt and PKC in the cardioprotection triggered by H2S postconditioning. To further confirm the role of Akt and PKC in the cardioprotection induced by H2S postconditioning, specific inhibitors of PI3K/Akt and PKC were administered in both SPostC and SPostC2 groups. As shown in Figs. 9 and 10, the inhibition of either PI3K/Akt pathway with LY (15 μM) or PKC with Che (10 μM) during the H2S infusion period significantly reversed the protective effect on contractile function in both SPostC and SPostC2 groups. These data suggest that the activation of both PI3K/Akt and PKC pathways are essential in the cardioprotection induced by both SPostC and SPostC2.

DISCUSSION

The main objective of this study was to unveil the role of endogenous H2S in the cardioprotection of IPostC and to explore the feasibility of postconditioning with exogenous H2S to trigger cardioprotection. Cardiodynamics of isolated perfused rat heart and myocardial infarction were chosen as the parameter to assess the functional performance of the heart.
after ischemia. In the present study, we found that IPostC significantly improved the heart contractile function after ischemia. This is consistent with the previous findings that IPostC protects the heart from lethal ischemia-reperfusion injury when assessed by infarct size (6, 22, 54), cardiodynamic performance (5, 11, 21, 62), cellular injury index (40, 47), etc. The inhibition of endogenous H2S synthesis with PAG, an irreversible inhibitor of CSE, partly attenuated the cardioprotective effect of IPostC. More importantly, we found that IPostC treatment stimulated the activity of H2S-generating enzymes in the early phase of reperfusion. These observations suggest a role for endogenous H2S in the phenomenon.

Interestingly, the inhibition of endogenous H2S synthesis with PAG did not block all signaling pathways stimulated by IPostC. PAG attenuated the IPostC-induced translocation of PKC-ε and PKC-δ/H9251 but failed to affect the phosphorylation of Akt, eNOS, and the translocation of PKC-δ. These data suggest a central dominant role for endogenous H2S in the activation of the prosurvival PKC-ε and PKC-α, whereas the activation of Akt, eNOS, and PKC-δ by IPostC might not be solely dependent on endogenous H2S. It has been reported that adenosine
receptor activation (34) or prolonged transient acidosis during IPostC is able to induce Akt phosphorylation. These signaling mechanisms may act in a concerted manner to trigger Akt activation and its downstream target eNOS to protect heart against ischemia-reperfusion injury via inhibiting the opening of the mitochondrial permeability transition pore (44).

In the present study, two IPostC protocols commonly used by other groups (24, 43) were employed to investigate whether postconditioning with exogenous H2S can also produce cardioprotection. Both protocols of H2S postconditioning produced significant and similar cardioprotective effects on both cardio-dynamic performance and myocardial infarction. These data suggest that interference of the early phase of reperfusion may produce strong cardioprotection against ischemic injury. Interestingly, the signaling mechanisms for the cardioprotection induced by the two protocols are not totally same. In addition to the common signaling mechanisms that both SPostC and SPostC2 stimulated Akt and PKC-ε, each of them activated different individual signaling mechanisms. SPostC, the six intermittent administrations of H2S, was able to activate PKC-ε and PKC-δ, whereas SPostC2, continuous infusion of H2S for 2 min, initiated eNOS phosphorylation. These data suggest that the signaling mechanisms for postconditioning are complicated. Proper manipulation of the early phase of postconditioning may have important clinical implications.

Over the decades, the Akt pathway has become such a target due to its role as a signaling pathway where the modulation of substrates prevents apoptosis. Akt functions as a survival kinase by phosphorylating a number of apoptosis-regulatory molecules such as bcl-xl/bcl-2-associated death promoter, forkhead transcription factors, caspase-9, and inhibitory-κB kinase to regulate NF-κB and GSK-3β (26). Notably, several studies have suggested that H2S may protect hearts from myocytes apoptosis (15, 37), which could be correlated with our data that SPostC/SPostC2 induced Akt activation. Therefore, the antiapoptotic effect of H2S may contribute significantly to the cardioprotection exerted by SPostC/SPostC2 through Akt activation.

Although in the present study we demonstrated that the cardioprotection induced by IPostC and SPostC involves Akt, eNOS, and PKC pathways, the effectors of this postconditioning are still unknown. There is a general trend that suggests that mitochondrial ATP-sensitive K+ channel (KATP channel) opening is one of the downstream effectors of IPostC (28, 33, 55). Previous studies have shown that the blockade of KATP channels attenuated the protective effect of H2S preconditioning (2, 30). More importantly, H2S was shown to be a direct KATP channel opener in vascular smooth muscle cells (42, 58) and insulin secreting cells (53). As such, the opening of KATP channels by H2S could possibly serve as one of the downstream effectors of H2S postconditioning. In addition, H2S has been shown to scavenge H2O2 in vitro (12), therefore to protect neuronal cells from oxidative stress (20) and hearts from ischemia injuries (12). Moreover, H2S has been shown to

Fig. 9. Effect of SPostC on cardiodynamics upon inhibition of Akt or PKC. A: experimental protocols: SPostC, LY-294002 (LY; phosphatidylinositol 3-kinase inhibitor), and chelerythrine (Che; PKC inhibitor). B: mean data of LVeDP (top left), LVDP (top right), +dP/dt (bottom left), and −dP/dt (bottom right). All values are means ± SE (n = 5 or 6). *P < 0.05, SPostC vs. SPostC-LY; #P < 0.05, SPostC vs. SPostC-Che.
attenuate myocardial ischemia-reperfusion injury by preserving mitochondrial function (10). In this context, the large amount of release H2S during IPostC may protect the heart from ischemia-reperfusion injury partly via inhibiting reactive oxygen species (ROS) generation (40), oxidant-mediated injury (22), and the preservation of mitochondrial function (10), all of which may also contribute to the effects of SPostC and SPostC2.

In the current study, we used constant flow in our isolated heart model. At the beginning of reperfusion, coronary perfusion pressure increases less and slower in the constant-flow model compared with the constant-pressure model, which reaches the selected/baseline value immediately (32). Unlike the constant-pressure model, this prevents the reactive hyperemia due to sudden increase in pressure that occurs during reperfusion after the global ischemia. This would result in a lower production of ROS and eventually result in less myocardial injuries (35). Indeed, the IPostC showed a greater infarct-sparing effect in the constant-flow than in the constant-pressure model (32). Therefore, it would be better to test the involvement of ROS in the cardioprotection of H2S postconditioning using a constant-flow isolated heart model.

We previously demonstrated that H2S may induce intracellular acidification in aorta smooth muscle cells (23). However, the intracellular pH regulatory effect may not play a major role in the cardioprotection induced by SPostC and SPostC2 in the current study. This is because H2S-induced intracellular acidification can only be achieved after the administration of H2S for 5 min (23), which is much longer than the perfusion period of H2S used in the current study.

We have demonstrated in our previous publications that endogenous H2S may participate in IPC since the application of PAG could essentially abrogate the protective effect of IPC. This is alike to the observation obtained in the case of IPostC. Similarly, exogenous H2S could serve as a pharmacological agent to induce IPC (H2S preconditioning) in both the immediate (2) and delayed (30, 31) window. The mechanisms involved have been intensively studied by several groups, including the opening of sarcolemmal KATP channels, PKC (δ-, α-, ε isoforms) activation, NO synthase activation (2, 31), heat shock protein 71 (3), ERK, and PI3K/Akt pathways (17). In the present study, we showed that PKC, PI3K/Akt, and eNOS are involved in H2S postconditioning, indicating that the mechanisms are highly similar to those stimulated by H2S preconditioning.

Taken together, we demonstrate for the first time that endogenous H2S plays an important role in modulating the cardioprotective effects of IPostC. Postconditioning with exogenous H2S is able to resume heart contractile functions to a similar extent as those produced by IPostC probably via the activation of the PKC and/or Akt/eNOS pathway. The effect of H2S postconditioning against ischemia-reperfusion injury has...
provided a firm ground to support H2S as a cardioprotective gasotransmitter.

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GRANTS

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